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Aquatic Toxicology 52 (2001) 101–115

AQUATIC TOXICOLOGY

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Induction and suppression of cytochrome P450 1A by 3,3′,4,4′,5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*)

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Received 25 April 2000; received in revised form 6 June 2000; accepted 6 June 2000

Abstract

The planar polychlorinated biphenyl (PCB) 3,3′,4,4′-tetrachlorobiphenyl (TCB) causes dose-dependent induction and post-transcriptional suppression of hepatic cytochrome P450 1A (CYP1A) in the marine teleost scup (*Stenotomus chrysops*). That suppression is linked to inhibition and oxidative inactivation of CYP1A by TCB. Other planar PCBs, including 3,3′,4,4′,5-pentachlorobiphenyl (PeCB), inactivate scup CYP1A in vitro leading us to hypothesize that PeCB also will suppress CYP1A in vivo. We examined induction and suppression of CYP1A by PeCB in scup, as related to oxidative stress. PeCB at a low dose (0.01 mg/kg) induced hepatic microsomal spectral P450 and CYP1A protein and catalytic activities (ethoxyresorufin *o*-deethylase (EROD) and methoxyresorufin *o*-demethylase (MROD)) over an 18 day period. A high dose (1 mg PeCB/kg) only minimally induced hepatic spectral P450 and CYP1A content, and EROD and MROD rates remained at control levels at all sampling times, while CYP1A mRNA expression was induced strongly (up to 35-fold) at both doses. High dose PeCB had minimal effects on content of P450A (a CYP3A protein), P450B (a CYP2B-like protein) and cytochrome b5 in scup liver, suggesting that the suppression was specific for CYP1A. High dose PeCB suppressed EROD but not CYP1A protein in the kidney but did not strongly suppress either CYP1A or EROD in the heart or gill. PeCB stimulated ROS production (oxidation of dihydroethidium) by liver microsomes from the low dose but not the high dose fish, and the rate of PeCB-stimulated ROS production was correlated with EROD activity ($r^2 = 0.641$, $P < 0.0005$). Oxidative stress, indicated by increased levels of catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase activities, was stimulated in the liver by low dose but not high dose PeCB. The results support a hypothesis that many PHAH can inactivate teleost CYP1A in vivo, and that CYP1A is a source of ROS. However, there appears to be a complex

Abbreviations: AHR, aryl hydrocarbon receptor; CYP, cytochrome P450; EROD, ethoxyresorufin *o*-deethylase; HCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl; HE, dihydroethidium; MROD, methoxyresorufin *o*-demethylase; PeCB-3,3′,4,4′,5-pentachlorobiphenyl; PHAH, planar halogenated aromatic hydrocarbon; ROS, reactive oxygen species; TCB, 3,3′,4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8, tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

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PII: S0166-445X(00)00141-7

balance between the effects of PeCB on the levels of active CYP1A, ROS release and oxidative stress.

Keywords: Cytochrome P450 1A; 3,3′,4,4′,5-Pentachlorobiphenyl; Oxidative stress; *Stenotomus chrysops*

1. Introduction

Planar halogenated aromatic hydrocarbons (PHAH) induce the expression of cytochrome P450 1A (CYP1A) genes via interaction with the aryl hydrocarbon receptor (AHR) (Hankinson, 1995). There is also a suppression of CYP1A in animals or in cells exposed to high doses of some PHAH. Suppression of CYP1A protein and activity has been reported in liver of the fish scup (*Stenotomus chrysops*) exposed to high doses of 3,3′,4,4′-tetrachlorobiphenyl (TCB) (Gooch et al., 1989; White et al., 1997) and in liver of rainbow trout and in chick embryo hepatocytes exposed to high doses of 3,3′,4,4′,5,-pentachlorobiphenyl (PeCB) (Rodman et al., 1989; Newsted et al., 1995) or 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB) (Miranda et al., 1990; Lorenzen et al., 1997). Recently, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was shown to cause a suppression of CYP1A1, but not CYP1A2, in mice (Shertzer et al., 1998).

Suppression of CYP1A by TCB in scup liver occurs at a post-transcriptional level. Thus, TCB given at 0.1 mg/kg induced hepatic CYP1A mRNA expression, protein and catalytic activity to nearly maximal levels, while a dose of 5 mg TCB/kg strongly induced CYP1A mRNA expression but minimally induced CYP1A protein and/or ethoxyresorufin *o*-deethylase (EROD) activity (Gooch et al., 1989; White et al., 1997). At least two mechanisms appear to be involved in that effect of TCB. In vitro, TCB is a competitive inhibitor of CYP1A and the lack of EROD induction in fish given high doses of TCB could result from inhibition by TCB retained in microsomes during subcellular fractionation (Gooch et al., 1989). The loss of CYP1A protein, on the other hand, appears to involve an oxidative inactivation of the enzyme. Scup liver microsomes incubated in vitro with TCB and NADPH show an irreversible loss of EROD capacity (Schlezinger et al., 1999) and loss of spectrophotometrically detected cytochrome P450 (White et al., 1997; Schlezinger et al., 1999). That loss of EROD is oxygen dependent and linked to enhanced electron transfer and to very slow rates of TCB turnover by CYP1A, consistent with uncoupling of electron transport from substrate oxidation (Schlezinger et al., 1999). Uncoupling apparently results in the formation of a reactive species in the active site, which inactivates the enzyme and which in vivo could target the enzyme for rapid elimination.

Interactions like those between CYP1A and TCB defined in vitro occur as well with other PHAH that are AHR agonists. PeCB and HCB competitively inhibit scup liver microsomal EROD and uncouple CYP1A in vitro, causing an inactivation that is time- and NADPH-dependent (Schlezinger et al., 2000b,c). In contrast, *ortho*-substituted PCB congeners that lack vicinal *meta–para* chlorines are not AHR agonists, and interact little with CYP1A. 2,2′,4,4′,5,5′-Hexachlorobiphenyl does not induce CYP1A and does not inhibit CYP1A (Gooch et al., 1989; Besselink et al., 1998). Likewise, 2,2′,5,5-tetrachlorobiphenyl does not inhibit or inactivate scup CYP1A (Schlezinger et al., 2000c).

Given the similarities between TCB and other PHAH in their effects on CYP1A in vitro, we hypothesized that those other planar PCB congeners also will suppress CYP1A in vivo at a post-transcriptional level, in patterns similar to that caused by TCB. In this study we examined PeCB for temporal and dose-dependent patterns of effect on expression of CYP1A mRNA, protein and activity in scup. Among the coplanar PCB congeners PeCB is the most potent in eliciting toxic effects in fish (Janz and Metcalfe, 1991; Walker and Peterson, 1991; Newsted et al., 1995) and mammals (Safe, 1990; Tillitt et al., 1991). PeCB also is the congener that is the most potent in vitro inhibitor of CYP1A of other fish species (Besselink et al., 1998). Based on its greater potency in those effects we hypothesized further that

PeCB would suppress CYP1A in vivo at doses lower than those at which TCB suppresses CYP1A. A further objective was to determine whether suppression of CYP1A in vivo occurs in extra-hepatic organs; studies to date have examined suppression of CYP1A only in the liver. In addition to examining suppression of CYP1A, we determined whether PCB treatment induced the capacity of liver microsomes to release reactive oxygen species (ROS), and whether the expression of antioxidant enzymes was stimulated in PeCB-treated fish. ROS are released from the active site of CYP1A during interaction with TCB (Schlezinger et al., 1999), potentially contributing to oxidative stress that could be involved in toxicity of these compounds.

2. Materials and methods

2.1. Chemicals

7-Ethoxyresorufin and dihydroethidium (HE) were purchased from Molecular Probes (Eugene, OR). PeCB (IUPAC # 126) was from Ultra Scientific (North Kingstown, RI). All other reagents were the highest grades available from Sigma (St. Louis, MO).

2.2. Animals

Scup (*S. chrysops*) were caught by trapping in Vineyard Sound, MA in August, 1996, and experiments were conducted in August, 1997. Fish were held in flow-through seawater tanks at 14°C and maintained on a diet of Purina trout chow, fed once weekly. Experimental animals (mixed sex) were gonadally undeveloped and ranged in size from 136 to 327 g. Fish were given single intraperitoneal injections with corn oil or solutions of PeCB in corn oil and sampled at various times. The volume of corn oil injected per mg body weight was the same for all fish. For each dose group there were three tanks each containing six fish. At each time point two fish were sampled from each tank yielding an $N = 6$ per group and avoiding pseudo-replication. The doses were 0.01, 0.1 or 1 mg (0.03, 0.3 or 3 μmol) PeCB/kg body weight. Untreated fish were killed by cervical transection on day 0. Control fish and fish treated with 0.01 or 1 mg (0.03 or 3 μmol) PeCB/kg body weight were killed 3, 7, and 16 or 18 days after treatment to assess time-dependent responses at a low and a high dose. Fish treated with 0.1 mg PeCB/kg were sampled on day 7 to assess dose-dependent responses at that time. Body and liver weights were used to calculate the hepatosomatic index (HSI = the percent of total body weight represented by the liver). Sections of liver for RNA preparation were removed and frozen in liquid N_2. Immediately following dissection of the heart, gill, kidney and liver, microsomal and cytosolic fractions were prepared from each organ as previously described (Stegeman et al., 1979), and frozen in liquid N_2 until use. Protein content was determined using the bicinchoninic acid method using bovine serum albumin as a standard.

2.3. Cytochromes b5 and P450

The content of hepatic microsomal P450 was determined from the dithionite-difference spectra of CO-treated microsomes (Bonkovsky et al., 1984). Cytochrome b5 content was determined from NADH difference spectra as previously described (Stegeman et al., 1979).

2.4. Quantification of CYP1A mRNA

RNA was prepared using RNA-Stat (Tel-Test, Friendswoods, TX) by following the manufacturer's instructions. RNA pellets were washed once in 4 M LiCl to remove glycogen, followed by two washes in 95% ethanol. Total RNA was quantified by its absorbance at 260 nm on a Shimadzu UV-2401PC spectrophotometer. RNA (10 μg each) was denatured in formamide/formaldehyde and separated on a 1% agarose/2.2 M formaldehyde gel, in 20 mM Mops running buffer. RNA was transferred to a positively charged membrane (Chomczynski, 1992) and loading per lane was determined to be equivalent by visualization of ethidium bromide staining of ribosomal RNA. The dried membrane was prehybridized for 2 h at 42°C, in a buffer composed of 5 × SSPE, 0.1% SDS, 2 × Denhardt's solution,

50% formamide and 100 μg/ml calf thymus DNA. ^{32}P-labeled probe (see below) was added to the pre-hybridization buffer, and the membrane was hybridized at 42°C for approximately 12 h, washed twice at 42°C for 30 min with 2 × SSC, 0.1% SDS and exposed to Kodak X-OMAT AR film. Relative amounts of CYP1A mRNA were determined by densitometric analysis of a video image (NIH Image 1.60b5).

A cDNA probe containing 75% of the scup CYP1A coding region (Morrison et al., 1995) was used for quantification of CYP1A mRNA. The probe was radiolabeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) using a Prime-a-Gene labeling kit (Promega, Madison, WI).

2.5. Western blotting for CYP1A quantification

For CYP1A determinations, liver microsomal protein was applied to a nylon membrane using a slot manifold (Kloepper-Sams and Stegeman, 1994). For all other analyses, microsomal proteins (30–40 μg) were resolved on 6–18% SDS-PAGE gels and electrophoretically transferred to nylon membrane. The primary antibodies were the mouse monoclonal antibody 1-12-3 (Park et al., 1986) against scup P450E or CYP1A (Morrison et al., 1995), a rabbit polyclonal antibody against scup P450B (a CYP2B-like protein) (Gray, 1988; Stegeman et al., 1990) and a rabbit polyclonal antibody against scup P450A (a CYP3A-like protein) (Celander et al., 1996). The secondary antibodies were alkaline phosphatase linked goat anti-mouse IgG and goat anti-rabbit IgG (Schleicher&Schuell, Keene, NH). Blots were developed with enhanced chemiluminescence (Tropix, Bedford, MA) and the signal quantified by video image analysis (NIH Image 1.60b5). CYP1A was quantified by comparison to the signal obtained with scup CYP1A standards.

2.6. Enzyme assays and superoxide formation

Microsomal EROD and MROD activities were determined fluorometrically by the method of Hahn et al. (1993), using a Cytofluor 2300 (Millipore) multiwell plate reader. Microsomal NADPH-cytochrome P450 reductase activity was assayed as previously described (Stegeman et al., 1982). The activities of cytosolic catalase (Beutler, 1975), glutathione peroxidase (Sies et al., 1979), glutathione reductase (Sies et al., 1979) and superoxide dismutase (McCord and Fridovich, 1969) were determined according to the methods cited.

Superoxide ($O_2^{-\bullet}$) formation by liver microsomes was measured using the fluorescent dye, HE, as described previously (Schlezinger et al., 1999). Additions to the incubations included 0.1 mg microsomal protein, acetone (5 μl), PeCB (1.7 nmol in acetone) and NADPH (1.4 mM). Microsomal ROS production stimulated by PCB 126 was calculated by subtracting the fluorescence in wells containing acetone from that in wells containing PeCB.

2.7. Statistics

Statistics were calculated using SuperAnova for Macintosh (Abacus Concepts, Berkeley, CA). Nested, one-factor ANOVAs were used to analyze differences between treatment groups within sampling days. There were no tank effects in liver P450 or enzyme results. Because RNA and extrahepatic tissue samples were pooled per tank, one-factor ANOVAs were used to analyze the results from these samples. The Tukey–Kramer multiple-comparisons test was used to determine differences between treatments. Due to deaths in the 1 mg/kg dose group remaining fish in this group were killed on day 16 while those in the control and 0.01 mg/kg dose groups were killed on day 18. Fish killed on days 16 and 18 were analyzed together. Data are reported as means ± S.E.

3. Results

3.1. Gross effects

No change in the hepatosomatic index occurred in any dose group over the experimental period (data not shown). There were no deaths caused by toxicity in the first seven days post-injection. However, one of five fish in the 0.01 mg PeCB/kg dose group died between days 7 and 18, and three of the six fish in the 1 mg PeCB/kg dose group

died between days 7 and 16. While toxicity could be involved in those deaths, that is not certain.

3.2. Hepatic monooxygenase components

Total P450 content in hepatic microsomes from control fish tended to decrease over time after treatment, but the change was not statistically significant. The content of P450 in liver microsomes of fish treated with 0.01 mg PeCB/kg was significantly greater than control levels on day 7 and on day 18 (Fig. 1A). The maximal induction of P450 by 0.01 mg PeCB/kg was 3-fold. Total P450 content was also induced by 0.1 mg PeCB/kg on day 7, and the specific content was similar to that induced by 0.01 mg PeCB/kg. In fish given 1 mg PeCB/kg there was no change in liver microsomal P450 content until day 16, when there was a 2-fold increase relative to control levels.

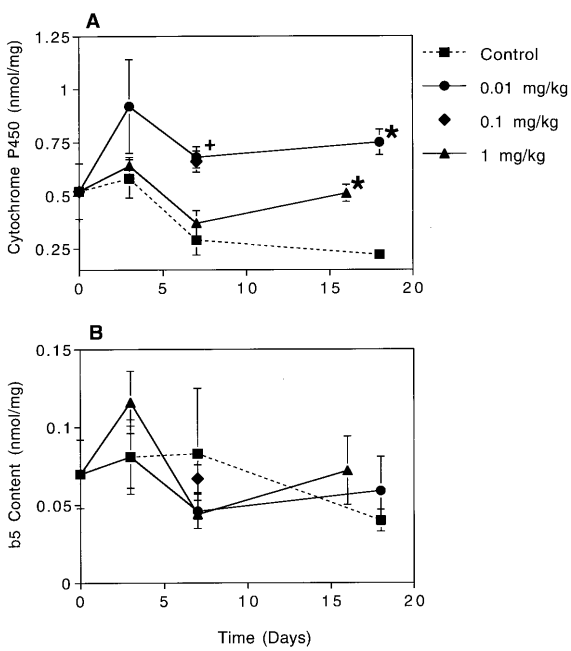

Fig. 1. Effect of PeCB on hepatic microsomal cytochrome P450 (A) and cytochrome b5 (B) content. P450 and b5 contents were determined spectrally as described previously (Bonkovsky et al., 1984). Data are the means ± S.E. of measurements on six fish per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose on day 16 ($n = 3$). *, Statistically different from control ($P < 0.05$). +, Statistically different from control and 1 mg/kg dose ($P < 0.05$).

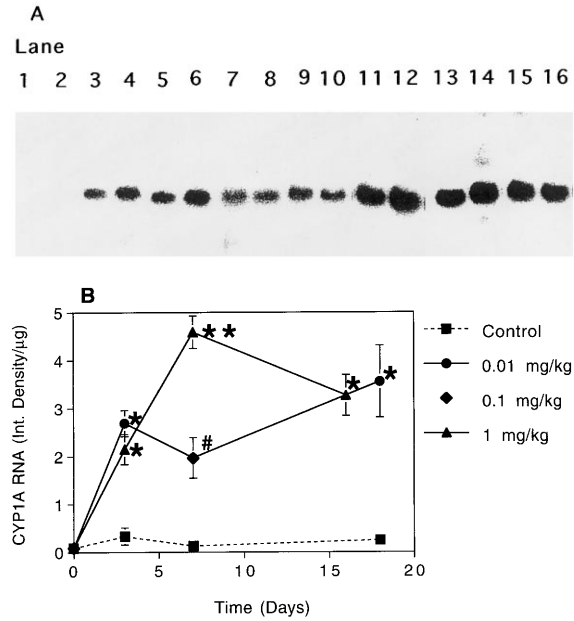

Fig. 2. Effect of PeCB on CYP1A mRNA expression in scup liver. (A) Northern blot showing detection of CYP1A mRNA, analyzed as described in Section 2. For each time point, livers from fish in the same tank were pooled before RNA preparation. Total RNA (10 µg/lane) was resolved on agarose gel, transferred to nylon membrane, probed with a ^{32}P-labeled cDNA containing 75% of the scup CYP1A coding region. Lanes 1–2, control, day 3. Lanes 3–4, 0.01 mg/kg, day 3. Lanes 5–6, 1 mg/kg, day 3. Lanes 7–8, 0.01 mg/kg, day 7. Lanes 9–10, 0.1 mg/kg, day 7. Lanes 11–12, 1.0 mg/kg, day 7. Lanes 13–14, 0.01 mg/kg, day 18. Lanes 15–16, 1.0 mg/kg, day 16. (B) Comparison of CYP1A mRNA in liver of scup treated with various doses of PeCB. Northern results were densitometrically quantified as in Section 2. Data are means ± S.E. of measurements on three pools per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose on day 16 ($n = 3$). Statistically different from control at $P < 0.05$ (*) or at $P < 0.01$ (**). #, Statistically different from control and 1 mg/kg dose ($P < 0.05$).

The specific content of cytochrome b5 was not affected by any of the treatments (Fig. 1B). The constant levels of hepatic microsomal cytochrome b5 with all treatments and at all times suggest that heme availability was not suppressed.

3.3. CYP1A mRNA

Hepatic CYP1A mRNA content was assessed by Northern blotting (Fig. 2A) using a cDNA probe containing 75% of the scup CYP1A coding

region. Ethidium bromide staining of ribosomal RNA on the membrane confirmed that there was equivalent loading of samples (data not shown). CYP1A mRNA content was significantly elevated by day 3 in both the 0.01 and 1 mg PeCB/kg dose groups and in both groups the levels remained elevated through days 16–18 (Fig. 2B). Maximal induction (15-fold) by 0.01 mg PeCB/kg occurred 18 days post-injection, and maximal induction (35-fold) by 1 mg PeCB/kg occurred 7 days post-injection. PeCB at 0.1 mg/kg also induced CYP1A mRNA content at day 7 (Fig. 2B).

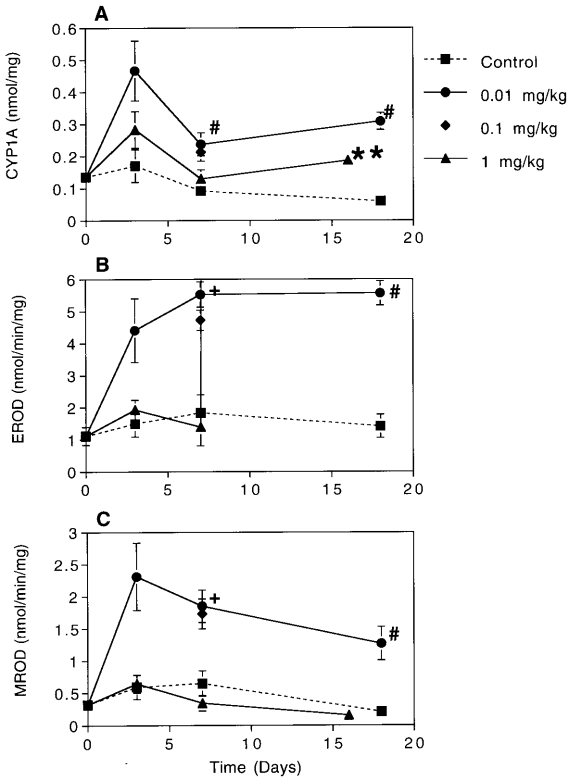

Fig. 3. Effect of PeCB treatment on hepatic microsomal CYP1A content (A) and catalytic activity (EROD-B; MROD-C). Liver microsomal fractions prepared from scup treated with corn oil, 0.01 mg PeCB/kg, 0.1 mg PeCB/kg or 1 mg PeCB/kg (0.2–4 μg/band) were applied to a nylon membrane with a slot blot manifold, and immunoblotted with MAb 1-12-3 against scup CYP1A. CYP1A reactive bands were visualized using enhanced chemiluminescence and quantified by densitometric analysis. Enzyme activities were determined fluorometrically by the method of Hahn et al. (1993)). Despite being in captivity for one year, the presence of measurable CYP1A in controls suggests that these fish were not depurated fully of inducing compounds accumulated in the wild. Data are the means ± S.E. of measurements on six fish per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose group on day 16 ($n = 3$). **, Statistically different from control ($P < 0.01$). +, Statistically different from control and 1 mg/kg dose group ($P < 0.05$). #, Statistically different from control and 1 mg/kg dose ($P < 0.01$).

3.4. CYP1A protein and activity

Induction of hepatic microsomal CYP1A protein followed a pattern similar to that of total P450 content. A peak in CYP1A content occurred on day 3 following the 0.01 mg PeCB/kg dose; however, the induction was significant only on days 7 and 18 (Fig. 3A). The 0.1 mg/kg dose significantly induced CYP1A protein at day 7, and the content was similar to that induced by the 0.01 mg/kg dose. Fish given 1 mg PeCB/kg showed no significant difference in hepatic CYP1A protein content relative to that in control values on days 3 or 7 after treatment. On day 16 CYP1A protein content was induced in the 1 mg PeCB/kg group although the content of CYP1A and fold induction were less than those in the 0.01 mg PeCB/kg group at that time. (We note that the fold induction of CYP1A protein or activity by PeCB was less than the fold induction by TCB reported by White et al. (1997); the fish in this study had not been depurated as long as those used by White et al., and levels of CYP1A in controls were higher.)

In fish treated with 0.01 mg PeCB/kg, hepatic microsomal EROD activity increased by day 3, reached a maximum by day 7 and remained at that level through day 18 (Fig. 3B). In those same samples MROD activity peaked on day 3 and then declined slowly through day 18 (Fig. 3C). The 0.1 mg PeCB/kg dose also significantly induced EROD and MROD activity at day 7 (Fig. 3B and C). However, neither EROD nor MROD activity were induced by the 1 mg PeCB/kg dose at any sampling time over the course of the experiment (Fig. 3B and C).

Overall, the content of total P450 in liver microsomes was correlated strongly with the content of immunodetected CYP1A (Fig. 4A). Determining the correlation between EROD rates and

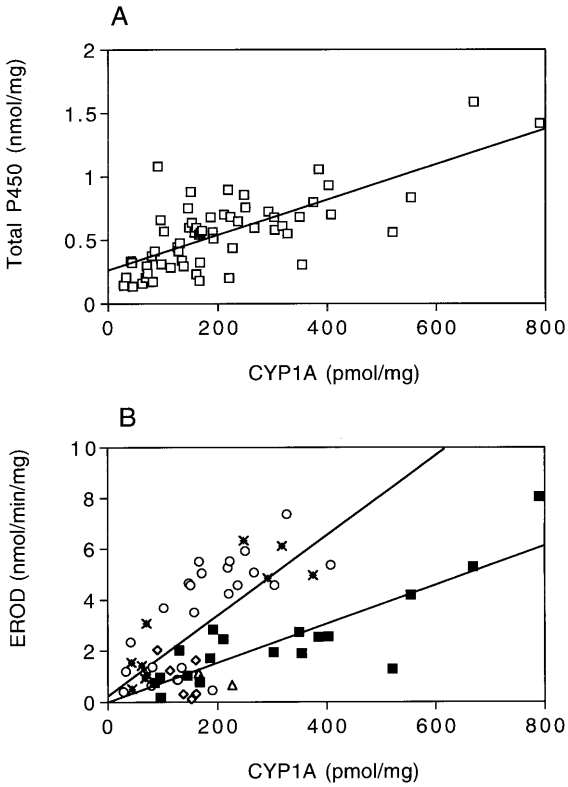

Fig. 4. Correlation of CYP1A with total P450 and EROD activity. (A) Microsomal P450 content vs. CYP1A content. The results obtained with all samples are regressed, without distinguishing between dose or time. $r^2 = 0.583$, $N = 61$; $P < 0.001$. (B) EROD CYP1A vs. EROD. Data for all samples were regressed, which revealed two groupings, those from fish sampled at day 3, and those from fish sampled at all other sampling times. In (B) filled squares are day 3 samples; open diamonds, day zero, circles, day 7, triangles, day 16, and $\times$, day 18. Regression analysis were performed separately for the two clusters. For day 3, $r^2 = 0.845$, $N = 18$; $P < 0.001$. For the other times, $r^2 = 0.522$, $N = 43$; $P < 0.001$.

CYP1A content revealed that the points fell into two groups with the data at the 3 day sampling time showing a different relationship than at the other times. The correlation determined for the samples at day 3 had a slope that was less than that obtained for the later sampling times (Fig. 4B), indicating that the turnover number for EROD activity (units/nmole CYP1A) had increased at the later times. EROD and MROD rates were strongly correlated at day 3 ($r^2 = 0.951$) but on days 7–18 the correlation was characterized by $r^2 = 0.746$, and with a lower slope (data not shown).

3.5. Other CYP proteins

Hepatic microsomes were immunoblotted with antibodies to two other major P450 forms expressed in scup liver, P450A and P450B. P450A is an apparent CYP3A (Celander et al., 1996) and P450B is a probable CYP2B (Gray, 1988; Stegeman et al., 1990). The content of P450A was not affected by any of the treatments (Fig. 5A). There also was little change in P450B content with treatment (Fig. 5B), although on day 7 the fish given 0.01 mg PeCB/kg had slightly elevated P450B content, and the fish given 1 mg PeCB/kg had

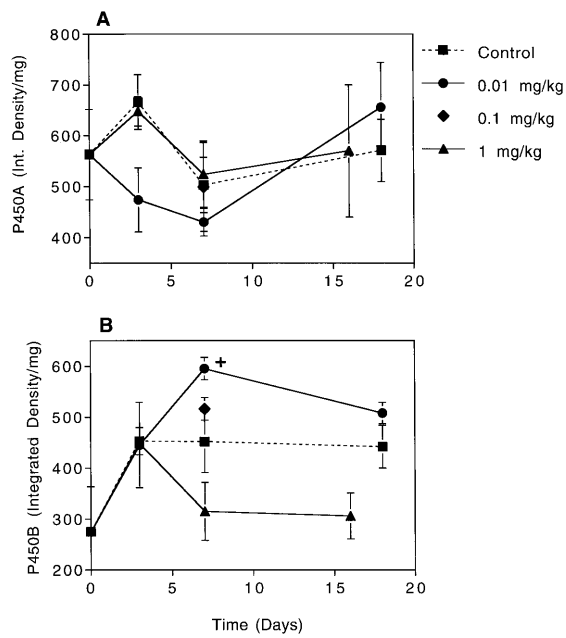

Fig. 5. Effect of PeCB treatment on hepatic microsomal P450A (panel A) and P450B (panel B). Liver microsomal fractions prepared from scup (30 μg/lane) were electrophoresed, transferred to nylon membrane, and immunoblotted with polyclonal anti-scup P450A (a CYP3A-like protein) and polyclonal anti-scup P450B (a CYP2B-like protein). Reactive bands were visualized using enhanced chemiluminescence and quantified by densitometric analysis. Data represent the means ± S.E. of measurements on six fish per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose on day 16 ($n = 3$). +, Statistically different from control and 1 mg/kg dose ($P < 0.05$).

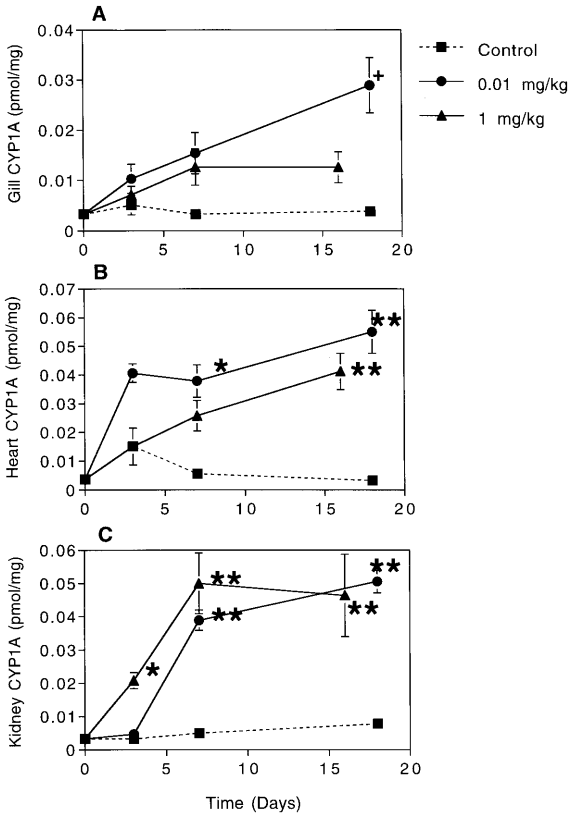

Fig. 6. Effect of PeCB treatment on extra-hepatic microsomal CYP1A content. (A) Gill. (B) Heart. (C) Kidney. For each time point, organs from fish in the same tank were pooled before microsome preparation. Microsomal fractions prepared from scup treated with corn oil, 0.01 mg PeCB/kg, 0.1 mg PeCB/kg or 1 mg PeCB/kg (30–40 μg/lane) were electrophoresed, transferred to nylon membrane, and immunoblotted with MAb 1-12-3 against scup CYP1A. CYP1A reactive bands were visualized using enhanced chemiluminescence and quantified by densitometric analysis. Data represent the means ± S.E. of measurements on three pools per treatment group. *, Statistically different from control ($P < 0.05$). **, Statistically different from control ($P < 0.01$). +, Statistically different from control and 1 mg/kg dose ($P < 0.05$).

slightly suppressed P450B content. P450B content in the 0.01 mg PeCB/kg group was significantly different from the 1 mg PeCB/kg group on day 7. These data and the lack of effect on cytochrome b5 indicate that the PeCB effect is largely specific for CYP1A, although the difference in putative CYP2B content between high and low dose fish suggests that other known CYP forms might be affected.

3.6. Extrahepatic organs

The patterns of CYP1A protein and EROD induction in microsomes from extra-hepatic organs of PeCB treated fish showed similarities and differences compared to the patterns seen in liver.

3.6.1. Gill (Fig. 6A and Fig. 7A)

In the 0.01 mg PeCB/kg group gill CYP1A content was induced at all sampling times. There also was a trend toward increased CYP1A content in the 1 mg PeCB/kg group on days 7 and 16.

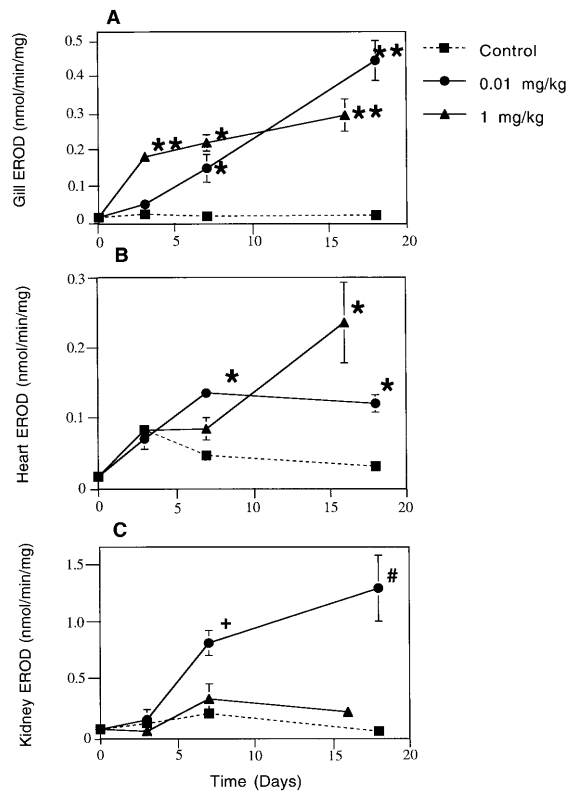

Fig. 7. Effect of PeCB treatment on extra-hepatic microsomal CYP1A catalytic activity (EROD). (A) Gill. (B) Heart. (C) Kidney. For each time point organs from fish in the same tank were pooled before microsome preparation. EROD activity was determined fluorometrically by the method of Hahn et al. (1993)). Data represent the means ± S.E. of measurements on three pools per treatment group. *, Statistically different from control ($P < 0.05$). **, Statistically different from control ($P < 0.01$). +, Statistically different from control and 1 mg/kg dose ($P < 0.05$). #, Statistically different from control and 1 mg/kg dose ($P < 0.01$).

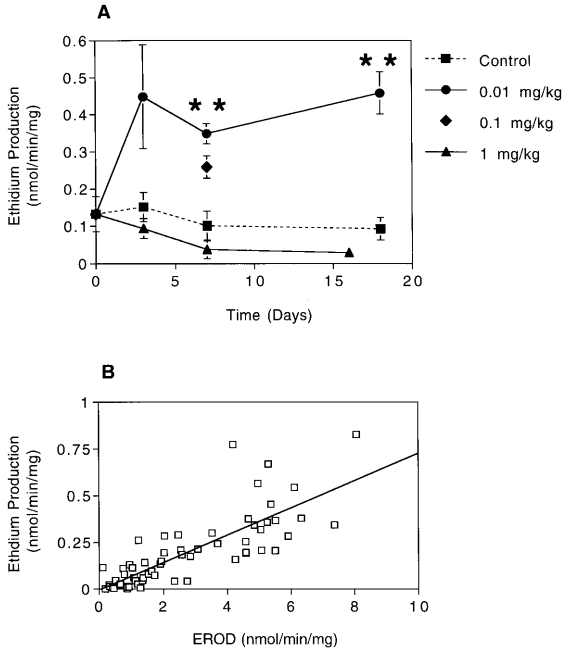

Fig. 8. ROS production by hepatic microsomes from PeCB-treated scup. (A) Effect of PeCB treatment on the potential of hepatic microsomal to produce ROS and (B) correlation of ROS production with EROD activity. Superoxide production was determined fluorometrically by monitoring the oxidation of dihydroethidium to ethidium as previously described (Schlezinger et al., 1999). Ethidium bromide was used as a standard. ROS production stimulated by PCB 126 was calculated by subtracting the fluorescence in the well containing acetone to that in the well containing PeCB. In (A), the data represent the means $\pm$ S.E. of measurements on six fish per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose on day 16 ($n = 3$). **, Statistically different from control ($P < 0.01$). In (B) ethidium production was related to EROD with $r^2 = 0.641$, $N = 61$, $P < 0.0005$.

Gill microsomal EROD activity was induced significantly by both doses of PeCB, with a 10- to 14-fold maximal induction. As with CYP1A, EROD rates in the 1 mg PeCB/kg group appeared to plateau through day 16.

3.6.2. Heart (Fig. 6B and Fig. 7B)

Cardiac microsomal CYP1A content was induced significantly by 0.01 mg PeCB/kg at day 7 and was induced further at day 18. The 1 mg PeCB/kg dose also induced CYP1A in the heart, significantly so at day 16. In the heart EROD activity was significantly induced by both the 0.01 mg PeCB/kg and the 1 mg PeCB/kg doses. At the later sampling times the induction was stronger in the high dose than in the low dose group.

3.6.3. Kidney (Fig. 6C and Fig. 7C)

In the kidney there was a significant induction of CYP1A content in the 1 mg PeCB/kg group by day 3. On day 7 and days 16–18 the levels of renal CYP1A were similarly induced in the 0.01 and 1 mg PeCB/kg groups. The 0.01 mg PeCB/kg dose significantly induced kidney microsomal EROD activity on days 7 and 18 (20-fold induction). However, there was no elevation of EROD activity in the 1 mg PeCB/kg dose group at any sampling time.

3.7. Oxidative stress

Binding of PeCB to scup CYP1A in vitro stimulates the production of ROS (Schlezinger et al., 2000b,c). Therefore, we examined liver microsomes from fish treated with PeCB for the capacity to produce ROS, and to determine whether microsomal ROS production could be stimulated by PeCB in vitro. The potential to produce ROS was determined by incubating hepatic microsomes from the various treatment groups with PeCB, NADPH and HE, which is oxidized to fluorescent ethidium by superoxide (Robinson et al., 1994). HE oxidation rates were greatest with microsomes from fish treated with 0.01 mg PeCB/kg (Fig. 8A). As with CYP1A content the potential to produce ROS was significantly elevated by day 7 in the 0.01 and 0.1 mg PeCB/kg groups and it remained elevated in the 0.01 mg PeCB/kg group through day 18. Adding PeCB to liver microsomes from the 1 mg PeCB/kg group did not stimulate ROS formation by those microsomes. Overall, the potential to produce ROS was highly correlated with EROD rates that were measured in these same samples (Fig. 8B).

We also assessed liver samples for indicators of oxidative stress by measuring the activities of several anti-oxidant enzymes, including catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase. Similar patterns of change were seen with all of these anti-oxidant enzymes

(Fig. 9). The rates of catalase, glutathione peroxidase and superoxide dismutase were significantly induced in the 0.01 mg PeCB/kg dose group at day 3 and then decreased to control levels by day 18. Glutathione reductase showed the same pattern of change although the differences were not significant. In contrast to this effect of the low dose of PeCB, in fish given 1 mg PeCB/kg none of these anti-oxidant enzymes showed any difference from rates in control fish over the course of the experiment.

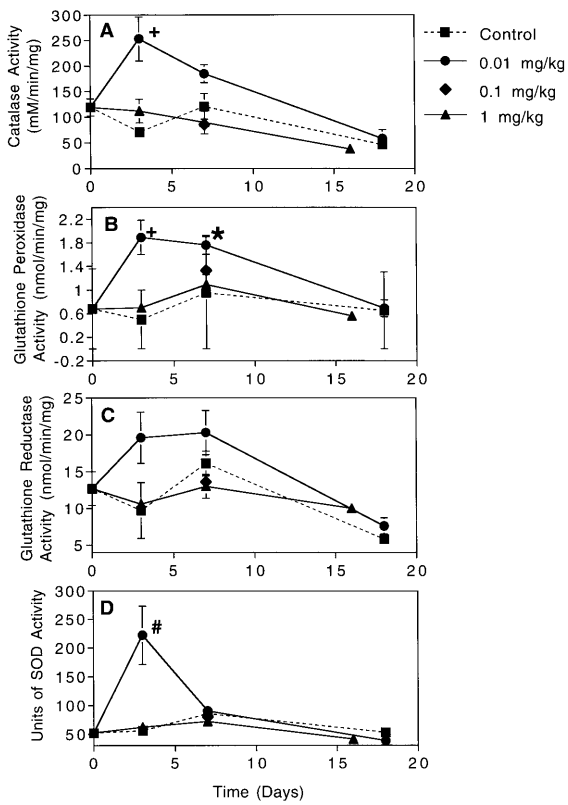

Fig. 9. Effect of PeCB treatment on activities of hepatic cytosolic catalase (A), glutathione peroxidase (B), glutathione reductase (C), and superoxide dismutase (D). Enzyme activities were quantified spectrophotometrically as previously described (McCord and Fridovich, 1969; Beutler, 1975; Sies et al., 1979). Data represent the means ± S.E. of measurements on six fish per treatment group except for the 0.01 mg/kg dose on day 18 ($n = 4$) and the 1 mg/kg dose on day 16 ($n = 3$). *, Statistically different from control ($P < 0.05$). +, Statistically different from control and 1 mg/kg dose ($P < 0.05$). #, Statistically different from control and 1 mg/kg dose ($P < 0.01$).

4. Discussion

CYP1A mRNA and protein were induced in scup liver by a low dose of PeCB while a high dose induced CYP1A mRNA but suppressed CYP1A protein and activity. The suppression of CYP1A by high-dose PeCB thus is a post-transcriptional effect, similar to the effect of TCB (White et al., 1997). PeCB and TCB inactivate scup CYP1A in vitro (Schlezinger et al., 2000c) by an oxidative mechanism related to uncoupling of the enzyme (Schlezinger et al., 1999). Thus, PeCB and TCB appear to suppress hepatic CYP1A in vivo by the same mechanisms, involving inhibition and oxidative inactivation. CYP1As from fish, birds and mammals are susceptible to uncoupling by TCB in vitro (Schlezinger et al., 2000b), and studies suggest that CYP1As in other species are susceptible to suppression by high doses of PeCB (Rodman et al., 1989; Newsted et al., 1995) or HCB (Miranda et al., 1990; Lorenzen et al., 1997). CYP1A inhibition, inactivation and ROS production are likely to occur with many PHAH and in vertebrate species generally.

4.1. Comparison of induction and suppression of hepatic CYP1A by PHAH

Dose–responses for CYP1A induction in scup liver have been determined for TCB (Gooch et al., 1989; White et al., 1997), 2,3,7,8-TCDF (Hahn and Stegeman, 1994), and now for PeCB. Induction of hepatic CYP1A mRNA levels by all three PHAHs is sustained for 14 days or more, ostensibly due to persistence of these inducers. Of the chemicals tested, TCDF is the most potent inducer of CYP1A mRNA or protein, maximally inducing at a dose of 0.01 μmol/kg. Maximal induction by PeCB occurred at 0.03 μmol/kg, in the range seen with lake trout (0.02 μmol PeCB/kg) (Palace et al., 1996) and rainbow trout (0.17 μmol PeCB/kg) (Newsted et al., 1995). TCB elicited maximal induction at a dose of 0.34 μmol/kg. Similar relative differences in potency of these PHAH are seen in mammals (Safe et al., 1985). At non-suppressive doses all three compounds induced scup liver CYP1A to the same peak levels, suggesting a similar efficacy in vivo. TCDF, PeCB

and TCB have similar efficacies for CYP1A induction in PLHC cells (Hestermann et al., 2000).

The suppression of hepatic EROD by higher doses of PeCB could involve inhibition of CYP1A by this PHAH retained in microsomes prepared from contaminated organs, as occurs with TCB (Gooch et al., 1989). TCB and PeCB are competitive inhibitors of EROD in scup, flounder and rat (Gooch et al., 1989; Besselink et al., 1998). The lack of CYP1A protein induction also contributes to the lack of EROD induction in fish given higher doses of TCB or PeCB.

There is a distinction between in vivo and in vitro effects of TCB or PeCB on CYP1A protein. Fish given the high doses of PeCB or TCB have little or no immunodetectable CYP1A in liver, while in vitro, PeCB or TCB (plus NADPH) quickly (30 min) cause a near total loss of EROD activity and substantial losses of spectral P450 from induced liver microsomes, but no loss of immunodetectable CYP1A (White et al., 1997; Schlezinger et al., 1999, 2000c). The loss of active CYP1A corresponds to the loss of spectral P450 and appears to involve both destruction of the heme and modification of the heme environment by the inactivating species formed in the active site, but without loss of the 1-12-3 epitope (Schlezinger et al., 1999). When fish CYP1As are inactivated and even proteolytically cleaved in vitro, the proteins retain the epitope recognized by MAb 1-12-3 (Goksøyr et al., 1991), yielding an immunodetected signal. In vivo the same inactivation could target the protein for rapid destruction, perhaps by ubiquitination, to fragments no longer bearing the 1-12-3 epitope.

In contrast to TCB and PeCB, TCDF did not suppress CYP1A in vivo (Hahn and Stegeman, 1994). The highest dose of TCDF in that study was 100-fold less than the inactivating doses of PeCB, similar to the difference between the low (non-inactivating) and high (inactivating) doses of PeCB. Assuming that TCDF also can uncouple scup CYP1A, the TCDF concentrations reached in vivo could be too low to inactivate more than a fraction of the CYP1A protein. Palace et al. (1996) also did not see inactivation of CYP1A in lake trout exposed to PeCB at 0.09 μmol/kg, 30-fold less than the dose that suppressed CYP1A in scup liver.

At days 3 and 7 hepatic CYP1A levels were completely suppressed by the high dose of PeCB, but by day 16 there was a modest reversal, and CYP1A was induced in that group. Scup given high dose TCB showed a similar reversal of suppression, by day 14 (White et al., 1997). In the TCB-treated fish that reversal was associated with a decrease in the content of TCB in the liver (White et al., 1997). With TCB, CYP1A protein and EROD both began to rebound, while with PeCB the EROD levels remained suppressed, even though the PeCB dose was 5-fold lower than the high dose of TCB. PeCB could be eliminated more slowly than TCB (Yoshimura et al., 1987; Koga et al., 1990; Coristine et al., 1993), but analysis of PeCB residues is required to confirm that.

As with TCB (White et al., 1997), PeCB effects were largely specific for CYP1A. The data suggest, however, that another CYP may be involved. In the low dose fish EROD rates peaked at day 7 and were undiminished by day 18, while total P450 and immunodetected CYP1A content both showed a peak at day 3. The discrepancy between CYP1A and EROD is consistent with the apparently lower turnover number for EROD in the day 3 fish. On the other hand, the peaks in P450 and CYP1A content coincided with a peak in MROD activity on day 3, after which MROD rates declined. EROD and MROD are catalyzed preferentially by CYP1A1 and CYP1A2 in mammals (Tsyrlov et al., 1993). In scup, a single CYP1A has been identified (Morrison et al., 1995), which catalyzes both EROD and MROD (Stegeman et al., 1996). Yet, multiple CYP1A genes have been identified in other fish and a second CYP1A could exist in scup. Possibly, a distinct CYP that also is recognized by MAb 1-12-3 and that preferentially catalyzes MROD activity was induced on day 3 and then declined, while a CYP more efficiently catalyzing EROD continued to increase. CYP1B, recently identified in scup (Godard et al., 2000), also might contribute to the different profiles for EROD and MROD. Further studies are required to address these issues.

4.2. Extrahepatic organs

CYP1A protein and activity in the heart or gill were not strongly suppressed by PeCB. This could result if lower concentrations of PeCB were reaching the active site of CYP1A or if uncoupling were less efficient in those organs than in the liver. Transfer of electrons to CYP1A by reductase is essential for uncoupling and oxidative inactivation by TCB (Schlezinger et al., 1999). Inactivation of CYP1A by TCB occurs more slowly in microsomes of gill and heart than in liver microsomes (unpublished data), which could result from less efficient electron transfer to P450. CYP1A in heart and gill microsomes is catalytically less efficient than in liver microsome (Miller et al., 1989; Stegeman et al., 1989), apparently due to insufficient reductase to support full activity of CYP1A (Stegeman et al., 1982).

In contrast to the other organs, in the kidney CYP1A was induced by both low and high doses of PeCB, but EROD was induced only at the low dose and was suppressed at the high dose. In scup treated with 5 mg TCB/kg both EROD and CYP1A protein were suppressed in the kidney (White, 1994). CYP1A is equally susceptible to in vitro inactivation in kidney and liver microsomes (unpublished results). The difference between TCB and PeCB effects on the kidney thus may reflect the 5-fold difference in the high doses of TCB and PeCB. Correspondingly less PeCB would reach the kidney and the modest difference between the $K_{inhibit}$ (0.02 μM) and K_{Intact} (0.14 μM) for PeCB inhibition and inactivation of CYP1A (Schlezinger et al., 2000b,c) might then lead to suppression of EROD but not CYP1A protein.

4.3. CYP1A and oxidative stress in vivo

The correlation between the capacity to catalyze EROD and the capacity for ROS production stimulated by PeCB supports the conclusion (Schlezinger et al., 1999) that PHAH-bound and uncoupled CYP1A is a source of ROS. ROS from CYP1A (Schlezinger et al., 1999) could stimulate the antioxidant response. The low dose of PeCB caused an increase in anti-oxidant enzyme activity in the liver that corresponded to the increased capacity of microsomes from those low dose fish to produce ROS. Increased anti-oxidant enzyme activity has been detected in other fish exposed to TCB or PeCB (Otto and Moon, 1995; Palace et al., 1996).

Mechanisms of planar PCB toxicity still are unclear. Metabolic activation of planar congeners is so slow as to be inconsequential. However, studies continue to show that PHAHs induce oxidative stress in mammals and other vertebrates, stimulating lipid peroxidation (Combs and Scott, 1975; Kato et al., 1981; Dogra et al., 1988; Hoffman et al., 1996), DNA damage (Stohs et al., 1990; Shertzer et al., 1998), and toxicity (Poland and Glover, 1980) that appear to be AHR dependent (Faux et al., 1992; Smith et al., 1995). Whether CYP1A-derived ROS contribute substantially to such effects is not known. There are sources of ROS additional to CYP1A including xanthine oxidase, peroxisome proliferation or mitochondrial multiplication (Stohs, 1990), and some PCB congeners can be metabolized to redox-cycling quinones (Oakley et al., 1996).

Changes in oxidative stress enzymes were not observed in the high dose fish. If, as we conclude, PeCB in high dose fish binds and inactivates a majority of CYP1A holoenzyme then there would be little active CYP1A available to produce ROS. As PeCB concentrations decrease in the liver a point may be reached at which CYP1A begins to accumulate, suggested by the rise in CYP1A observed at 16 days post-injection in the high dose fish. Such accumulating CYP1A could participate in ROS production and effects of that ROS might ensue. Notably, at the 16-day sampling time these same fish showed a PeCB dose-dependent activation of NF-κB in the liver (Schlezinger et al., 2000a); NF-κB is a transcription factor that is activated by ROS.

One might ask whether suppressive effects of planar PCBs occur in the environment, and whether the production of ROS during the interaction of PHAH with CYP1A might contribute to the toxicity of PHAHs in the environment. Complete suppression of CYP1A in scup liver occurred at a concentration of 2 μg TCB/g wet weight of tissue (Gooch et al., 1989). TCB con-

centrations as high as 0.4 μg/g dry weight (or approximately 0.04 μg/g wet weight) have been measured in wild caught fish (Elskus et al., 1994). In some environments the sum of coplanar congeners might reach levels inhibitory to CYP1A, as suggested in winter flounder from New Bedford Harbor (Monosson and Stegeman, 1994), and repeated exposures could shift the balance to inhibition and possibly inactivation (Monosson and Stegeman, 1991). However, the anti-oxidant response occurred at PeCB doses 10–100 times lower than the suppressive doses, and thus environmental levels of planar PCBs could well elicit biologically significant uncoupling and ROS release. At what point this might lead to toxicity in liver or other organs is not clear.

In summary, this study establishes that a suppression of CYP1A in scup liver occurs with high doses of PeCB, as it does with TCB, and probably represents a general phenomenon associated with uncoupling of CYP1A by PHAH. While the loss of CYP1A could reduce activation of some PAH, other consequences are unknown. Some studies have indicated a regulatory relationship between CYP1A and the AHR which may be perturbed by the loss of CYP1A (Chang and Puga, 1998). Fish with suppressed levels of CYP1A also may be used to examine the role of hepatic CYP1A in the disposition and toxicity of various chemicals, akin to the way that CYP1A knock-out mice might be used. The results in the high dose fish, and the different temporal patterns of CYP1A and antioxidant enzyme induction in low dose fish, suggest that PHAH exposure results in a complex interplay of promotion of and defense against oxidative stress and its relationship to toxicity. Suppression of CYP1A by high dose planar PCBs may provide a window into the mechanisms of PHAH toxicity.

Acknowledgements

This research was supported in part by EPA grant R827102-01-0, by NIH grant P42-ES07381, by the Air Force Office of Scientific Research (AFOSR) F40620-94-1039, by a WHOI Mellon Award and by the Lyons Fellowship at MIT (to J. Schlezinger). We gratefully acknowledge Dr Afonso Bainy, D.J. Struntz, Bruce Woodin, Eli Hestermann, and Agnes Knorr for occasional technical assistance and Dr Vicki Starczak for assistance with the statistical design. This is Contribution Number 10178 of the Woods Hole Oceanographic Institution.

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